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(54) Title: QUANTITATIVE TESTING FOR VITAMIN B12

(57) Abstract

The vitamin B12 (cobalamin) level of human blood and mammalian tissue is routinely assayed utilizing radioisotope dilution assay (RIDA) techniques. The presence of vitamin "B12 analogues" in such samples, which analogues are erroneously assayed as true vitamin B12 utilizing prior art RIDA techniques, has been determined. Such errors are due to the fact that binding proteins commonly used in prior art RIDA techniques normally include proteins which bind both true vitamin B12 and previously unrecognized vitamin B12 analogues. The errors caused by the B12 analogues are avoided by using a composition for binding vitamin B12 which is substantially free of substances which bind vitamin B12 analogues. Moreover, by using both types of binders in separate assays, the amount of analogue present may be arrived at by taking the difference of the assays.

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QUANTITATIVE TESTING FOR VITAMIN B_{12}

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to methods and materials for assaying mammalian blood and tissue. More specifically it relates to methods and materials for determining the amounts of vitamin B_{12} and vitamin B_{12} analogues in human plasma.

Prior Art

For many years it has been recognized that the assay of the vitamin B_{12} level in humans is a valuable technique for diagnosing and subsequently treating certain diseases, such as for example, pernicious anaemia, post gastrectomy states, nutritional deficiencies, intestinal disorders, and others. Initially, vitamin B_{12} was assayed microbiologically using either Euglena gracilis or Lactobacillus leichmannii. More recently, radioisotope dilution (RID) assays for B_{12} have been utilized. Such radioisotope dilution assay techniques are well documented in the literature, see for example Lau, et.al. (1965) "Measurement of Serum B_{12} Levels Using Radio-isotope Dilution and Coated Charcoal," BLOOD, 26, 202, as modified by Raven et.al. (1968) "The Effect of Cyanide Serum and Other Factors on the Assay of Vitamin B_{12} by Radio-Isotope Method Using ^{57}Co-B_{12}, Intrinsic Factor and Coated Charcoal," GUYS HOSPITAL REPORTS, 117, 89; and (1969) "Improved Method for Measuring Vitamin B_{12} in Serum Using Intrinsic Factor, ^{57}Co-B_{12} and Coated Charcoal," JOURNAL OF CLINICAL PATHOLOGY, 22, 205.

Such prior art radioisotope dilution assay of vitamin B_{12} generally includes the steps of freeing the endogenous B_{12} from its natural binding protein by boiling at a selected pH and then adding a measured amount of radioisotope ^{57}Co-B_{12} and a limited amount of binding protein. All of the binding protein will be bound by some form of B_{12} since the amount of radioisotope B_{12} added is, by itself, sufficient to bind the small amount of protein. As both the natural B_{12} and the radioactive B_{12} compete to bind with the protein, the degree to which the radioactive count of the protein bound B_{12} was

inhibited was thought to be indicative of the amount of natural B_{12} present in the sample undergoing testing.

More specifically, in the technique of Lau et.al. as modified by Raven et.al., serum B_{12} is separated from binding protein in the plasma sample by boiling with 0.25N HCl. Radioisotope B_{12} is added to the reaction mixture and the B_{12} mixture is reacted with protein, normally in the form of a commercially available binder. Then the free or unbound B_{12} is separated from the protein bound B_{12} by protein-coated charcoal and the radioactivity of the supernatant liquid containing the mixture of bound radioactive B_{12} and bound non-radioactive B_{12} counted for radioactivity. The serum B_{12} concentration is then calculated from the count, often by comparison with a standard chart. Almost as soon as this technique began to be utilized it was recognized that the vitamin B_{12} measurements it provided were usually inconsistent with the results obtained by other measuring techniques for B_{12}, such as the microbiological assays. Most often, the vitamin B_{12} assay obtained by radioisotope dilution techniques have been found to be high. Many theories have been advanced to explain the cause of the high vitamin B_{12} readings. However, it is believed that nowhere in the prior art is it recognized that there are substances in mammalian blood and tissue which react with certain non-specific protein binders in the radioisotope dilution assay technique to provide an analysis of vitamin B_{12} which is apparently higher than the amount of B_{12} actually in the sample. Additionally, it is believed that nowhere in the prior art is it recognized that most common and commercial RID assay protein binders are not specific to vitamin B_{12}, but that they are also capable of binding with the heretofore unknown B_{12} analogues and thus provide erroneous B_{12} assays.

BRIEF DESCRIPTION OF THE INVENTION

As has already been indicated, in the standard radioisotope binding assay for vitamin B_{12}, a known amount of radioactive vitamin B_{12} is mixed with a prepared to-be-tested sample. Then, a known, but extremely limited, amount

of protein which is capable of binding with both the natural and radioactive vitamin B_{12} is added to the mixture. Then, utilizing well known techniques, the radioactivity of the bound sample is compared, for example, with a standard curve to determine the amount of natural vitamin B_{12} present in the tested sample. Such standard curves are initially established for use in RID assay, for example, by measuring the amount of bound radioactive B_{12} in the presence of the same type and amount of protein binder, but with several different amounts of known non-radioactive B_{12}.

It has now been discovered, for what is believed to be the first time, that mammalian blood and tissue contain materials other than vitamin B_{12} which couple with certain binding proteins which are commonly used in RID assays. For purposes of this specification and claims the non-vitamin B_{12} materials which are capable of binding with such proteins will be herein referred to as "vitamin B_{12} analogues," "B_{12} analogues" or simply as "analogues." They are referred to as analogues, not due to their chemical structure, which is not known with certainty, nor in the commonly accepted chemical sense of the word "analogue." Rather they are referred to as analogues due to their reactivity with the binding proteins commonly used in RID assays. As will be shown in more detail, hereinafter, there are other similarities which have been discovered between vitamin B_{12} and the newly discovered analogues which are present in mammalian blood and tissue.

After the presence of B_{12} analogues was discovered it was then determined that protein binders commonly present in RID assays were: (1) Non-specific in binding to only vitamin B_{12}; and (2) reactive in binding with both vitamin B_{12} and B_{12} analogues; and (3) capable of reacting with both B_{12} and B_{12} analogues independent of pH. These are most commonly R proteins. Additionally, it has been determined that other protein binders, are: (1) Very specific in their reactivity substantially only with vitamin B_{12}; (2) substantially non-reactive with the B_{12} analogues; and (3) non-reactive with either vitamin B_{12} or B_{12} analogues

in highly acid environments. These are most commonly proteins in the form of pure human intrinsic factor (IF), hog IF, rabbit IF, other IFs and vitamin B_{12} specific binders.

In the past the problem has been that RID binders include substantial amounts of protein which is not specific to vitamin B_{12}. Therefore, the radioisotope dilution assay utilizing that binder on samples which contain B_{12} analogues will produce a measurement which indicates a greater amount of B_{12} present in the plasma than exists in fact. As will be shown in more detail hereinafter, commercially available protein binders, which have heretofore been labeled as containing intrinsic factor, in fact include only about 10% to about 30% intrinsic factor protein, while the balance of the protein in the binder is of a non-specific type, such as R protein. Thus, the protein materials in the commercial protein binders are capable of indiscriminate reaction with the heretofore unrecognized vitamin B_{12} analogue materials in mammalian blood and tissue. These extraneous reactions give RID analyses having the appearance of apparently higher vitamin B_{12} content than the samples in fact contain. This is due to the fact that when the binder includes protein which is non-specific to vitamin B_{12} and which is capable of reacting with both vitamin B_{12} and B_{12} analogues, then the use of this protein in the radiobinding assay measures both the vitamin B_{12} and the vitamin B_{12} analogues which are present in the sample. However, in accordance with the present invention, when the proteins which are utilized are substantially specific to vitamin B_{12}, such as substantially pure intrinsic factor, then in the RID assay one binds and measures substantially only the vitamin B_{12} in the sample, without the measurement of extraneous B_{12} analogues. This provides a more accurate vitamin B_{12} RID assay.

Based on these discoveries it is proposed that in the practice of RID assay only protein which is specific in its reaction to vitamin B_{12} be utilized. Alternatively, it is proposed that mixtures of vitamin B_{12} specific and

non-specific binding proteins be treated, for example, with an excess of material which will bind or inactivate only the non-specific binding proteins, such as vitamin B_{12} analogues, prior to its use in RID assays, so that the non-specific protein will be substantially unavailable for reaction with any vitamin B_{12} or analogues in a sample when the RID assay is conducted. In yet another modification of the present invention, crude binder, including non-specific binding proteins, is subjected to proteolytic enzyme treatment prior to utilization as a vitamin B_{12} binder in RID assays. Such proteolytic enzyme treatment destroys the binding ability of the non-specific proteins without destroying the binding ability of the proteins which are specific to vitamin B_{12}.

Utilizing the techniques of the present invention, the B_{12} analogues can by assayed by analyzing the amount of vitamin B_{12} present utilizing, for example, a vitamin B_{12} specific binder, then assaying the sample utilizing a non-specific binder and determining the difference between the two assays as a measure of the amount of vitamin B_{12} analogues present.

These and other techniques are readily determined, once, as taught for the first time by the present invention, the presence of B_{12} analogues in mammalian blood and tissue is recognized.

The foregoing and other objects, features and advantages of the invention will be apparent from the following more particular description of preferred embodiments of the invention.

DETAILED DESCRIPTION OF THE INVENTION

In the following examples and tables certain chemical components were utilized. For ease of communication they have been given shortened names in the text. The concordance between the "component" names and their actual compositions is as follows:

Components	Actual Composition
A. Buffer	1.0M Tris (hydroxymethyl) aminomethane-HCl pH 10.0

B. Albumin	Bovine serum albumin, 2 mg per ml in H_2O
C. Salt	0.15M NaCl
D. Boiled buffer	(1 part) 0.5M sodium acetate-HCl pH 4.5 (1 part) 0.01M KPO_4 pH 7.5, 0.15M NaCl (2 parts) 50 µg per ml KCN in 0.15M NaCl The complete solution is heated of 45 min. at 100°C.
E. Standard (100 pg/ml B_{12})	Solution D containing 100 pg per ml vitamin B_{12}. The solution is heated for 45 min. at 100°C after the vitamin B_{12} is added. The concentration of vitamin B_{12} in the stock solution used to make component E is determined by its light absorbance at 278, 361 and 550 nm.
F. Standard (1000 pg/ml B_{12})	Same as component E except that the vitamin B_{12} concentration is pg/ml.
G. (^{57}Co) B_{12}	1000 pg per ml of (^{57}Co) B_{12}, (150-300 µCi/µg), in H_2O.
H. Binder	Present in 0.01M Tris-HCl pH 8.2, containing 0.15M NaCl and 50 µg per ml bovine serum albumin. Binders are diluted in this solution to reach a concentration of 700 pg per ml of vitamin B_{12} binding ability.

Individual binders are as follows:

1) Human intrinsic factor (Human IF) -
Human gastric juice containing more than 95% intrinsic

factor based on assays employing inhibition of vitamin B_{12} binding with anti-intrinsic factor antibodies (95% inhibition) and cobinamide (5% inhibition).

2) Human R protein (Human R)- Human saliva containing more than 95% R protein based on assays employing inhibition of vitamin B_{12} binding with cobinamide (95% inhibition) and anti-intrinsic factor antibodies (5% inhibition)

3) Hog intrinsic factor (Hog IF) - This protein was purified from "Hog intrinsic factor concentrate" by affinity chromatography on vitamin B_{12}-Sepharose employing gradient elution with guanidine-HCl followed by gel filtration. The final preparation contained more than 95% intrinsic factor based on assays employing inhibition of vitamin B_{12} binding with anti-intrinsic factor antibodies (95% inhibition) and cobinamide (5% inhibition).

4) Hog R protein (Hog R) (Also designated in the scientific literature as Hog non-intrinsic factor concentrate)

This protein was purified from "Hog intrinsic factor concentrate" as described above in 3). The final preparation contained more than 95% R protein based on assays employing inhibition of vitamin B_{12} binding with anti-intrinsic factor antibodies (5% inhibition) and cobinamide (95% inhibition).

5) Rabbit intrinsic factor (Rabbit IF) - An extract of rabbit gastric mucosa containing more than 95% intrinsic factor based on assays employing inhibition of vitamin B_{12} binding with anti-intrinsic factor antibodies (95% inhibition) and cobinamide (5% inhibition).

6) Hog intrinsic factor concentrate (Hog IFC) - A crude extract of hog pyloric mucosa. It contained 25% Hog IF and 75% Hog R based on assays employing inhibition of vitamin B_{12} binding with anti-intrinsic factor antibodies (25% inhibition) and cobinamide (75% inhibition).

7) Hog IFC + Cobinamide - Hog IFC containing the vitamin B_{12} analogue cobinamide

([CN, OH] Cbi) in a molar amount equal to 100 times the total vitamin B_{12} binding ability, i.e. a 100 fold excess of cobinamide.

8) Hog IFC + CN-Cbl [bde-OH] - The same as item 7) above except that the analogue added is CN-Cbl [bde-OH] and is present in a 1000 fold molar excess.

9) Hog IFC + [3,5,6-Me_3BZA] (CN,OH)Cba - The same as item 7) above except that the analogue added is [3,5,6-Me_3BZA] (CN, OH)Cba.

10) Digested Hog IEC - Hog IFC incubated with bovine pancreatic trypsin (2 mg per ml) and bovine pancreatic chymotrypsin (2 mg per ml) for 60 min. at 37°C.

I. Charcoal — A solution containing 25 mg per ml neutral charcoal (Norit) and 5 mg per ml bovine serum albumin in H_2O.

J. unknown sample — Samples containing unbound vitamin B_{12} are diluted in solution D (see above). Samples containing bound vitamin B_{12}, such as serum, are prepared as follows:

(1 part) sample
(1 part) 0.5M sodium acetate-HCl pH 4.5
(2 parts) 50 µg per ml KCN in 0.15M NaCl

The complete mixture is heated at 100°C for 45 min. followed by centrifugation at 5000x g at 4°C for 20 min. The supernatant is removed and used for assay.

Each of the RID assays referred to herein utilized the components referred to above. The method and order of utilizing the components is that set forth in Table 1. That is, components A, B, C, etc. or the buffer, albumin, and salt, respectively, etc. were added in the order, from left to right, shown in Table 1. After the addition of ^{57}Co-B_{12} the components are mixed thoroughly to mix both the naturally occurring B_{12} and the radioisotope B_{12} to make them compete and equally available to react with the binder. After the addition of the binder, H, the components were again mixed thoroughly, and then incubated for 45 minutes at about 37°C. Charcoal was then added to the incubated mixture and the components again mixed thoroughly and incubated for another 5 minutes at room temperature. This was followed by centrifuging at 2000 x g at 4°C for 30 minutes. Then 1000 µl of the resulting supernatant liquid is pipetetted from the sample and a determination of the amount of ^{57}Co-B_{12} present is made. The amount of ^{57}Co-B_{12} is indicative of the amount of natural B_{12} in the tested sample, with lesser amounts of ^{57}Co-B_{12} being indicative of greater amounts of natural vitamin B_{12} in the sample.

Calculations of vitamin B_{12}, utilizing the datea obtained in the foregoing manner, is made as follows:

Calculation of data from radiobinding assay for plasma vitamin B_{12} assay as outlined in Table I

1) The values in tubes 3 and 4, the "blank" tubes without binder are averaged and subtracted from all other tubes starting with tube 5.
2) The background radiation is subtracted from tubes 1 and 2 and these values are averaged.
3) Tubes 5 and 6 are averaged. This value should be at

Table I

Flow Sheet for Radiobinding Assay for Vitamin B_{12}

Tube #	A Buffer (ul)	B Albumin (ul)	C Salt (ul)	D Boiled Buffer (ul)	E Standard 100pg/ml B_{12} (ul)	F Standard 1000pg/ml B_{12} (ul)	J Unknown Sample (ul)	G [^{57}Co] B_{12} (ul)	H Binder (ul)	I Charcoal (ul)
1,2	225	50	875	800	-	-	-	50	-	-
3,4	225	50	375	800	-	-	-	50	-	500
5,6	225	50	325	800	-	-	-	50	50	500
7	225	50	325	720	80	-	-	50	50	500
8	225	50	325	640	160	-	-	50	50	500
9	225	50	325	560	240	-	-	50	50	500
10	225	50	325	300	500	-	-	50	50	500
11	225	50	325	720	-	80	-	50	50	500
12	225	50	325	640	-	160	-	50	50	500
13	225	50	325	450	-	350	-	50	50	500
14	225	50	325	-	-	800	-	50	50	500
Unknown Sample	225	50	325	-	-	-	800	50	50	500

least 15% below the average value for tubes 1 and 2 to insure that all of the binder is saturated in the presence of (^{57}Co) B_{12} alone.

4) Values for each tube beginning with tube 7 are divided by the average of tubes 5 and 6 to give values for "% trace binding."

5) Percent trace binding for tubes 7-14 are used to obtain a standard curve. We plot % trace binding on the ordinate of logit-log paper versus pg vitamin B_{12} on the log scale.

6) The amount of vitamin B_{12} in unknown samples is determined by interpolation from the standard curve or data of % trace binding versus pg vitamin B_{12}.

7) The standard curves for all of the various binders used are virtually indistinguishable and vary little from day to day. Nevertheless, a complete standard curve is always obtained for every binder with each set of assays. Representative data obtained with the assays are present in Table II.

Evidence as to the Origin and Existence of Vitamin B_{12} Analogues in Mammalian Blood and Tissue

Once the problem of the prior art is recognized, that is, that there are vitamin B_{12} analogues present in mammalian blood and tissue, it becomes a relatively simple matter to prove the existence and chemistry of such analogues. It is also appropriate to prove that the various steps of the RID assay do not cause the B_{12} analogues to be formed, for example, from vitamin B_{12}.

In one instance this has been most convincingly shown by obtaining pure crystalline vitamin B_{12}, subjecting various known concentrations of it to the same conditions used to extract endogenous vitamin B_{12} from blood and tissue samples (boiling for 45 minutes in the same extraction solution) and then analyzing them by RID assay using several binding proteins, for example, in the form of human IF, hog IF, human R, hog R and hog IFC on different portions of the same extracted vitamin B_{12} samples.

Table II

Standard Curves obtained with the radiobinding assay for vitamin B_{12} using various binders

Vitamin B_{12} added[a] (pg)	% trace binding observed with various binders[b]				
	Human IF	Human R	Hog IF	Hog R	Hog IFC
0	(100)	(100)	(100)	(100)	(100)
8	90	93	94	89	89
16	85	84	83	83	80
24	79	78	74	76	76
50	64	60	58	56	59
80	45	44	44	43	43
160	30	28	28	27	28
350	15	14	15	14	14
800	6	6	7	6	7

(a) The vitamin B_{12} was boiled for 45 min. at 100°C in the same solution used to extract endogenous vitamin B_{12} from human plasma.

(b) Assays were performed on different days.

Referring to Table II, it will be seen that when various known amounts of pure vitamin B_{12}, ranging from about 8 pg to about 800 pg were tested with various protein binders, that in each instance, the percent of radioactive trace binding, or more accurately, the inhibition of ^{57}Co-B_{12} binding, observed was substantially the same for each binder. It is thus seen, that regardless of which protein binder is utilized, the percent binding, i.e. inhibition of the (^{57}Co)-B_{12} is substantially the same. This is indicative of the fact that during preparation for RID assay the pure vitamin B_{12} was not converted to analogues of the type which have now been observed in mammalian blood and tissue. It is also indicative of the fact that in the absence of interferring masking components in the samples, such as B_{12} analogues, any of the binding proteins can be utilized to provide substantially equally acurate RID assays of vitamin B_{12}. Furthermore, the data in Table II should be suitable as a standard in the determination of vitamin B_{12} by the same RID assay.

By comparison, when endogenous vitamin B_{12} was extracted from serum from 74 normal blood donors (37 women, 37 men, ages 17-61) and tested utilizing the same binding proteins with the exception of hog IFC which was not used, the results were quite different. In every case in which serum from normal donors was tested greater inhibition of ^{57}Co-B_{12}, and therefore greater apparent vitamin B_{12}, was observed with assays employing, as the binder, human R or hog R than was observed with assays employing human IF or hog IF. The data on the 74 normal donors is included in Table III. Other data concerning patients with diagnosed vitamin B_{12} deficiencies are present, and comparisons between the normal donors and patients have also been made on Table III, and will be discussed in more detail hereinafter. Referring to Table III it is seen that the mean endogenous vitamin B_{12} RID assay levels, in terms of pg of vitamin B_{12} per ml of serum, are 548 and 542 for human R and hog R,

Table III

Distribution of serum vitamin B_{12} values as measured with various binders for 74 normal subjects and 71 patients with clinical evidence of vitamin B_{12} deficiency
All assays were performed at pH 9.0

Serum Vitamin B_{12} (pg/ml)	Human R		Hog R		Human IF		Hog IF		Human R minus Human IF		Hog R minus Hog IF	
	Normals	Patients	Normals	Patients	Normals	Patients	Normals	Patients	Normals	Patients	Normals	Patients
0-24						8		11				
25-49						6		5				
50-74						5		3		1	2	1
75-99		1		1		2		2	2		3	
100-124		2		1					2	3	9	3
125-149		2		5	4		1		7	3	5	3
150-174		3		1	5		3		6	5	9	5
175-199		4		3	2		3		6	3	8	3
200-224	1	1		2	5		4		8	2	11	2
225-249		3	1	1	6		4		2	3	6	3
250-274				3	10		9		10		4	
275-299		1	2	1	4		3		5		1	
300-324	1	2	4	2	12		4		6		3	
325-349	3	1	2	1	5		6		4	1	5	1
350-374	5	1	1				10		4		1	
375-399	3		2		3		4		4		2	
400-424	5		3		4		5		5		1	
425-449	3		4		3		2		2			
450-474	4		6		2		3				1	
475-499	5		5				3				1	
500-524	7		4		1		3					
525-549	3		4		1							
550-574	5		3		2		1				1	
575-599	1		2		1				1			
600-624	2		4		1		3				1	
625-649	5		4									
675-699	2		3									
700-724	3		6				1					
725-749	2											
750-774	3		1				1					
775-799			1		1							
800-824			2									
825-849												
850-874	1		1									
875-899	1		2				1					
900-924	1		1									
925-949	2		1									
950-974	1											
975-999	1											
1000-1024												
1025-1049												
1050-1074	1											
1075-1099												
1100-1124												
1125-1149			1									
1150-1174												
1175-1199												
1200-1224												
1225-1249	1											
range of serum B_{12}	220-1230	85-355	245-1135	84-342	130-785	0-78	135-880	0-86	70-575	56-337	50-605	72-332
mean of serum B_{12} (a)	548		542		298	36	361	33	254	169	212	171
mean ± 2 Std. Dev.	282-1040		276-1065		136-656		157-717		70-661		58-531	

(a) Based on values obtained with log vitamin B_{12}. Log values were used since these gave a normal curve while the curve using untransformed values were skewed to the right.

respectively, but only 298 and 361 for human IF and hog IF, respectively. This demonstrates that something is present in extracts of normal human serum which inhibits the vitamin ^{57}Co-B_{12} binding ability of human R and hog R to a greater extent than it inhibits the binding ability of human IF and hog IF. Under current RID assay techniuqes the greater inhibition which is found using human R and hog R is analyzed to indicate a higher vitamin B_{12} content. It is those substances, which have now been found to be present in human blood serum and which preferentially inhibit ^{57}Co-B_{12} binding of human R and hog R, which have been herein denominated as "vitamin B_{12} analogues."

Chemical Nature and Properties of Vitamin B_{12} Analogues

The vitamin B_{12} analogues, which are herein for the first time identified as being present in mammalian blood and tissue, have been isolated by paper chromatography and compared with pure vitamin B_{12}. Vitamin B_{12} and the so-called "vitamin B_{12} analogues" were found to have the following properties in common: (1) Both were adsorbed to charcoal and remained adsorbed when the charcoal was washed with 5% phenol; (2) Both were eluted from charcoal when the charcoal was washed with 67% acetone; (3) Both were extracted from aqueous solution into phenol and remained in the phenol phase even when the phenol was washed repeatedly with water; (4) Both passed into the aqueous phase when the phenol layer was dissolved in an excess of diethyl ether; (5) Both eluted with similar apparent molecular weights (approximately 1356) during gel filtration on columns of Bio-Rad P-4 polyacrylamide; (6) Both were adsorbed to a column of Sepharose-2B agarose that contained covalently bound hog R protein and both remained bound when the column was washed with 0.1M glycine-NaOH pH 10.0, 1.0M NaCl, and both were eluted from the Sepharose with either 85% phenol or 60% pyridine. Because of these similarities the newly discovered material is seen to be similar to vitamin B_{12} and is thus referred to as vitamin B_{12} analogue.

The chemical nature and structure of the newly discovered vitamin B_{12} analogues which are now found to be present in mammalian blood and tissue is not known. An effort was made to compare them with chemically true forms of vitamin B_{12}, sometimes referred to in the literature as analogues of vitamin B_{12}, namely CN-B_{12}, OH-B_{12}, adenosyl-B_{12} and CH_3-B_{12}, already known to be present in serum and tissues. This was done by adding 500 pg of each of these four known forms of vitamin B_{12} to four different portions of the same human serum, in the dark. Prior to the additions the serum contained 250 pg and 450 pg of vitamin B_{12} as assayed by RID using human IF and human R, respectively, thus exhibiting a difference of 200 pg. After addition of the materials to the serum, each was allowed to incubate in the dark for 15 minutes to allow binding of the added known forms of vitamin B_{12} to the binding proteins normally present in the serum. Then the serum, with the added forms of vitamin B_{12} was extracted utilizing standard conditions and the apparent amount of vitamin B_{12} assayed by RID utilizing both human R protein and human IF protein. Both the human R and human IF assays showed an increase in the apparent amount of vitamin B_{12} of about 500 pg. However, the original difference observed between the values obtained with the human R protein and the human IF protein, i.e. 200 pg, did not change. If any of the added known forms of vitamin B_{12} in the human serum had been converted to the newly discovered analogues, then the assays would have shown an increase in the difference. This provides evidence that the newly discovered vitamin B_{12} analogues were not formed from any of the known endogenous forms of native viatime B_{12} during the extraction procedure.

Isolation of Vitamin B_{12} Analogue

The materials which are herein designated as "vitamin B_{12} analogues" and which have been found to preferentially inhibit R proteins in the vitamin B_{12} assays were substantially separated from endogenous vitamin B_{12} by the following purification scheme. A trace amount of 150 pg ^{57}Co-B_{12}, was added to 1800 ml of freshly collected normal human

plasma. The added $^{57}Co\text{-}B_{12}$ was sufficiently small that it did not interfere with subsequent RID assays. After incubating at room temperature for 30 minutes the vitamin B_{12} was extracted and assayed under standard conditions. When human IF binder was utilized in the RID assay the extract was found to contain 1050 ng of vitamin B_{12}, but when human R protein was utilized as the binder it appeared to contain 2030 ng of vitamin B_{12}, almost twice as much vitamin B_{12}. The extract was then passed through a column of Sepharose containing covalently bound hog R protein. The column retained greater than 99% of the $^{57}Co\text{-}B_{12}$ as well as the endogenous vitamin B_{12} as assayed by RID with human IF or human R protein. After the column was washed with a variety of buffers and water the material was eluted with 60% pyridine, taken to dryness under vacuum, dissolved in water, and adsorbed onto charcoal. The charcoal was washed with 5% phenol followed by water and the mixture of vitamin B_{12}, $^{57}Co\text{-}B_{12}$ and analogue B_{12} was eluted from the charcoal with 67% acetone. The material was again taken to dryness under vacuum, dissolved in water, and then separated utilizing 19 inch long Whatman 3MM paper for paper chromatography and a solvent system consisting of 800 ml sec-butanol, 8 ml glacial acetic acid, 6 mmol HCN and 400 ml water. The chromatography was performed in the descending manner for 30 hours at room temperature in an environment that inhibited evaporation of the solvent. The paper chromatogram was allowed to dry in a fune oven and divided into 38 one-half inch fractions and numbered, with fraction 1 starting at the point of application and number 38 being at the lowest point on the chromatogram. Each one-half inch fraction was then incubated with 5 ml of water at 4°C for twelve hours to elute the vitamin B_{12}, $^{57}Co\text{-}B_{12}$ and B_{12} analogues. The water was then removed and taken to dryness under vacuum. Each dried fraction was then dissolved in 2.5ml of water and assayed for $^{57}Co\text{-}B_{12}$ and for vitamin B_{12} using a variety of binding proteins. The final recovery of $^{57}Co\text{-}B_{12}$ was 64%. The apparent recoveries of vitamin B_{12} were 75% when using human IF in the assay

and 66% when using human R in the assay. The results of the assays employing the 38 fractions obtained by paper chromatography are presented in Table IVA, IVB, and IVC. Similar data concerning paper chromatography of ^{57}Co-B_{12} and pure vitamin B_{12}, for reference as a control, are presented in Table IVD. The data in these several chromatography tables summarized for convenience in Table IVE reveals that the behavior of ^{57}Co-B_{12} that was extracted from human plasma did not change its chromatographic behavior, and thus was not altered during the standard extraction procedure or any of the purification steps. In a similar manner it is postulated that true vitamin B_{12} is not altered in any of the purification or process steps of the assay.

Referring to the control chromatogram of Table IVD, it is seen that the several RID assays of pure vitamin B_{12} performed variously with human R protein, human IF protein and hog IFC gave substantially a single symmetrical peak of activity. In each instance greater than 95% of the vitamin B_{12} was found to be present in fraction 14 through 16. Similar results, as shown in Table IVB, were obtained from the paper chromatogram of the plasma extract when the binding protein was human IF, hog IF and rabbit IF. These data are an indication that these three IF binding proteins are substantially specific in their binding ability to vitamin B_{12}, and substantially non-reactive with vitamin B_{12} analogues present in plasma.

Efforts were made to modify hog IFC, which is a commonly used binder in RID assays and which has been found to contain as much as 90% hog R protein and as little as 10% hog IF, by removing or inactivating the hog R. In several instances the hog IFC was incubated with an excess amount of three chemically synthesized vitamin B_{12} analogues before it was utilized in the RID assay. Referring to Table IVC, it is seen that after this modification the chromatogram results obtained utilizing the modified hog IFC closely resemble the results obtained with substantially pure hog IF. It is therefore seen, that in the practice of the

Table IXA

[57Co] B_{12} and plasma B_{12} assayed after elution from paper chromatography. All assays were performed at pH 9.0 except where indicated.

Fraction #	[57Co] B_{12}		B_{12} assayed with various binders									
			Human R		pH 1.8 Human R		Hog R		pH 1.8 Hog R		Hog IFC	
	(cpm)	(%)	(ng)	(%)	(ng)	(%)	(ng)	(%)	(ng)	(%)	(ng)	(%)
1			2		1		1		1		1	
2												
3												
4			1		1		1		2		1	
5			2				2		3		2	
6			3		3		2		4		2	
7			4		4		4		5		3	
8			4		3		4		5		3	
9			7	1	6		6	1	8	1	5	1
10	300	1	24	2	29	2	26	2	29	2	16	2
11	500	2	42	3	54	4	36	3	49	3	26	3
12	300	1	25	2	34	2	26	2	38	2	19	2
13	200	1	31	2	44	3	30	3	55	4	22	2
14	4600	20	209	16	235	16	176	16	240	16	180	17
15	14000	61	562	42	615	43	461	41	576	37	474	46
16	2100	9	103	8	122	9	84	7	122	8	91	9
17	300	1	85	6	118	8	85	8	128	8	53	5
18	300	1	68	5	68	5	68	6	100	6	45	4
19	200	1	23	2	17	1	17	2	27	2	15	1
20			45	3	39	3	39	3	60	4	24	2
21			42	3	25	2	25	2	52	3	21	2
22			8	1	5		7	1	8	1	5	1
23			8	1	4		5		8	1	4	
24			7	1	4		5		7		3	
25			9	1	1		6	1	3		4	
26			16	1	1		10	1	4		5	1
27			5		1		3		2		2	
28			2				1		1		1	
29			4				2		1		2	
30			1				1				1	
31			1									
32-38												

Table IVB

Assay of [^{57}Co] B_{12} and plasma B_{12} after elution from paper chromatography. All assays were performed at pH 9.0.

Fraction #	[^{57}Co] B_{12}		B_{12} assayed with various binders					
			Human IF		Hog IF		Rabbit IF	
	(cpm)	(%)	(ng)	(%)	(ng)	(%)	(ng)	(%)
1			1				1	
2								
3								
4					1		1	
5					1			
6					1		1	
7					1		1	
8			1		1		2	
9	300	1	4	1	5	1	8	1
10	500	2	7	1	10	1	16	2
11	300	1	5	1	7	1	10	1
12	200	1	14	2	10	1	13	1
13	4600	20	169	21	169	22	170	19
14	1400	61	488	61	450	57	480	55
15	2100	9	73	9	72	9	87	10
16	300	1	17	2	25	3	42	5
17	300	1	11	1	15	2	25	3
18	200	1	3		5	1	5	1
19			5	1	7	1	8	1
20			2		4	1	4	
21								
22-38								

Assay of [^{57}Co] B_{12} and plasma B_{12} after elution from paper chromatography.
All assays were performed at pH 9.0.

Table 22

Fraction #	[^{57}Co] B_{12}		B_{12} assayed with various binders									
			Hog IFC		Hog IFC + Cobinamide		Hog IFC + CN-Cbl[bde-OH]		Hog IFC + [3,5,6-Me$_3$BZA] (CN, OH)Cbá		Hog IFC Digested	
	(cpm)	(%)	(ng)	(%)	(ng)	(%)	(ng)	(%)	(ng)	(%)	(ng)	(%)
1			1								1	
2												
3												
4			1								1	
5			2				1		1		1	
6			2				1		1		1	
7			3				1		1		2	
8			3				1		1		2	
9			5	1	1		1		2		3	
10	300	1	16	2	4		5	1	7	1	9	1
11	500	2	26	3	10	1	10	1	13	2	15	2
12	300	1	19	2	8	1	8	1	9	1	11	1
13	200	1	22	2	9	1	10	1	12	2	14	2
14	4600	20	180	17	174	21	170	22	148	20	153	19
15	14000	61	474	46	474	58	435	55	396	53	402	50
16	2100	9	91	9	83	10	81	10	80	11	84	10
17	300	1	53	5	25	3	27	3	34	4	36	4
18	300	1	45	4	16	2	19	2	21	3	27	3
19	200	1	15	1	4		4	1	6	1	9	1
20			24	2	7	1	7	1	11	1	13	2
21			21	2	3		4	1	7	1	9	1
22			5	1					1		2	
23			4						1		1	
24			3								1	
25			4								1	
26			5	1							2	
27			2									
28			1								1	
29			2									
30			1									
31-38												

Table IV D

Assay of [^{57}Co] B_{12} and native B_{12} after elution from paper chromatography. All assays were performed at pH 9.0.

Fraction #	[^{57}Co] B_{12}		B_{12} assayed with various binders					
			Human R		Human IF		Hog IFC	
	(cpm)	(%)	(ng)	(%)	(ng)	(%)	(ng)	(%)
1								
2								
3								
4								
5								
6								
7								
8								
9								
10	200	1	1				1	
11	600	2	1		1		2	1
12	400	1	1		1		2	1
13	400	1	4	1	3	1	6	2
14	6800	24	86	25	86	26	90	27
15	15800	56	210	60	198	60	189	56
16	3100	11	40	12	38	12	40	12
17	200	1	2	1	1		3	1
18	200	1	2	1	1		2	1
19	100		1				2	1
20-38								

Table IVE

Summary of the data in Tables IVA - IVD involving assays of [^{57}Co] B_{12} and B_{12} after elution from paper chromatography. Assays for B_{12} were performed at pH 9.0 except where indicated.

Sample Chromatographed	Item Assayed	Assay Binder	Chromatogram Fractions 1-13 (ng)	1-13 (%)	14-16 (ng)	14-16 (%)	17-38 (ng)	17-38 (%)	1-38 (ng)	1-38 (%)
[^{57}Co] B_{12} + B_{12}	[^{57}Co] B_{12}	-	-	5	-	92	-	2	-	100
"	B_{12}	Human R	7	2	336	97	5	1	348	100
"	B_{12}	Human IF	5	1	322	98	2	1	329	100
"	B_{12}	Hog IFC	11	3	319	95	7	2	337	100
[^{57}Co] B_{12} + plasma B_{12}	[^{57}Co] B_{12}	-	-	6	-	91	-	3	-	100
"	B_{12}	Human R	145	11	874	65	324	24	1343	100
"	B_{12}	Human R (pH 1.8)	179	12	972	68	283	20	1434	100
"	B_{12}	Hog R	138	12	721	64	274	24	1133	100
"	B_{12}	Hog R (pH 1.8)	199	13	938	61	402	26	1539	100
"	B_{12}	Hog IFC	100	10	745	72	185	18	1030	100
"	B_{12}	Human IF	31	4	730	91	38	5	799	100
"	B_{12}	Hog IF	37	5	691	88	56	7	784	100
"	B_{12}	Rabbit IF	53	6	737	84	84	10	874	100
"	B_{12}	Hog IFC + Cobinamide	32	4	731	89	54	7	818	100
"	B_{12}	Hog IFC + CN-Cbl[bde-OH]	47	6	624	83	81	11	752	100
"	B_{12}	Hog IFC + [3,5,6-Me$_3$BZA] (CN,OH)Cba	38	5	686	87	61	8	785	100
"	B_{12}	Hog IFC - Digested	60	7	639	80	102	13	801	100

present invention, mixtures of protein including both vitamin B_{12} specific binding protein and binding protein which is not specific to vitamin B_{12} can be modified by the addition of an excess amount of vitamin B_{12} analogue, by which process the analogue binds with the non-specific protein to render it substantially bound or inactive so that it is not available to react with vitamin B_{12} or (^{57}Co) B_{12} present in samples undergoing RID tests. The amount of vitamin B_{12} analogue to be added to a mixture of specific and non-specific proteins in order to bind or inactivate the non-specific proteins may vary over a wide range, depending on both the proteins which are present and the vitamin B_{12} analogues which are utilized as the binding or inactivating material. Generally speaking, for the examples shown in Table IVC, cobinamide may be added in an amount equal to that required for complete binding of the non-specific protein, up to an amount as much as ten million times greater than the amount needed to bind the protein, with the preferred range being about ten to about ten thousand times in excess of that required for complete binding. CN-Cb (bde-OH), known as CoB-cyano-cobamic a,c,g-triamide may be utilized in an amount at least about ten times to about ten million times in excess of the amount required to bind with the non-specific protein, with an amount of about one hundred to about one hundred thousand times excess being preferred. The (3,5,6-Me_3BZA) (CN,OH)-cba, known as Co (3,5,6-trimethylbenzimidazole) cobamide should also be utilized in amounts from about one to ten million times in excess of the amount of non-specific protein, with an amount in the range of about ten to about ten thousand times excess being preferred. Suitable amounts of other vitamin B_{12} analogues may be utilized in a similar manner to bind or inactivate non-specific proteins present in mixtures with specific proteins in order to obtain a preparation of binding protein which will substantially bind only ^{57}Co-B_{12}, or the vitamin B_{12} naturally present in the samples being tested, and thus

give a more accurate quantitative RID assay of vitamin B_{12} in samples undergoing tests.

Again, referring to Table IVC, data on samples of hog IFC digested with trypsin and chymotrypsin are shown. These and other proteolytic enzymes are specific in their ability to substantially digest R proteins while leaving intrinsic factor proteins unaffected and available as substantially the only protein for binding $^{57}Co\text{-}B_{12}$ and vitamin B_{12} in RID assays. Other enzymes, including, for example, elastase may be utilized for the same purpose. The amount of the enzymes utilized is in the range of about 0.01 to about 100 milligrams per mililiter of protein treated, with a preferred amount being about 0.05 to about 40 milligrams per mililiter of protein. Utilizing this proteolytic enzyme digestion process a protein binder is provided which substantially binds only $^{57}Co\text{-}B_{12}$ and vitamin B_{12} and is not affected by vitamin B_{12} analogues in the samples being tested and which therefore gives a more accurate RID assay than is obtained when utilizing the original mixture of hog IFC proteins which included non-specific proteins which would have been capable of reacting with the newly discovered vitamin B_{12} analogues in samples to give inaccurate assays as to the amount of vitamin B_{12} in test samples.

Now, referring to Table IVA, when samples from the plasma extract chromatogram were assayed for vitamin B_{12} with human R, hog R and hog IFC, different results were obtained than when those samples were assayed with human IF, hog IF, rabbit IF or hog IFC treated with vitamin B_{12} analogues or hog IFC digested with proteolytic enzymes. In each case where human R, hog R or untreated hog IFC were utilized as the binding protein the tests gave the appearance that more vitamin B_{12} was present in the chromatogram samples, especially in fraction 1 through 13 and 17 through 38. This observation, when taken with the above data, provides strong evidence that normal human plasma contains a number of vitamin B_{12} analogues that compete with $^{57}Co\text{-}B_{12}$, in significant amounts, for binding to R protein. It also indicates that such activity on the part of the B_{12}

analogues is substantially absent when the binding protein utilized in the RID assay is substantially specific to vitamin B_{12}.

It should also be noted, see Table IVA, that the chromatogram data suggests that the lack of specificity of human R and hog R is unchanged when RID assays are performed at acid pH. This indicates that erroneous results will be obtained for the true vitamin B_{12} content of samples which contain vitamin B_{12} analogues when RID assays are performed at acid pH.

Using the same techniques and criteria described above it has been discovered that vitamin B_{12} analogues are not only present in serum obtained from human blood, but are also present in mammalian tissues in even higher concentrations than they are in blood. Vitamin B_{12} analogues extracted from mammalian tissues have been purified using the same schemes as described above. When analyzed utilizing paper chromatography, they exhibited similar mobilities to those of the vitamin B_{12} analogues observed in the samples extracted from blood serum. Since larger amounts of the vitamin B_{12} analogues are present in tissue, they can be observed visually as red or orange spots during paper chromatography. The absorption spectra of vitamin B_{12} analogues purified from tissue extracts have been determined and demonstrate that they are similar to, but distinct from, the absorption spectrum of true vitamin B_{12}. These observations provide additional evidence that the materials in blood serum which preferentially react with R proteins and not intrinsic factor proteins are in fact varieties of vitamin B_{12} analogues.

The newly discovered vitamin B_{12} analogues also differ from vitamin B_{12} in terms of their biological activity. Thus, as shown in Table V, the serum vitamin B_{12} values obtained with Euglena gracilis for eleven patients diagnosed to have vitamin B_{12} deficiency were substantially similar to the results obtained by RID assay using human IF or hog IF as the binding protein. It is to be further noted, that all of the values obtained by either microbiologic assay or

Table V

Vitamin B_{12} levels in 21 patients with clinical evidence of vitamin B_{12} deficiency

Patient	Vitamin B12 assayed with various binders				Vitamin B_{12} assayed with Euglena Gracilis
	Human R (pg/ml)	Hog R (pg/ml)	Human IF (pg/ml)	Hog IF (pg/ml)	(pg/ml)
1	155	138	16	0	43
2	310	295	51	86	52
3	310	255	24	35	46
4	135	132	0	22	40
5	215	250	52	60	25
6	347	342	0	0	56
7	102	120	0	0	35
8	240	242	58	65	79
9	160	125	38	23	0
10	85	84	0	12	23
11	235	255	5	11	0
12	188	190	41	38	
13	178	192	48	38	
14	298	305	53	57	
15	355	310	78	82	
16	178	210	48	22	
17	128	140	31	8	
18	106	190	50	40	
19	163	155	0	0	
20	178	132	25	0	
21	230	215	75	41	
Mean (1-11)	209	203	22	29	36
Mean (1-21)	205	204	33	30	
Normal Range (mean ± 2 std. dev.)	220-1230	245-1135	136-656	157-717	(>130)
Number within normal range	9 (43%)	10 (48%)	0	0	0

by assay using human IF or hog IF were substantially lower than the values obtained when the RID assay was carried out utilizing human R or hog R as the binding protein. This indicates that the vitamin B_{12} analogues which have now been identified in mammalian blood and tissue do not possess vitamin B_{12} activity of the type which is required to promote the growth of Euglena gracilis.

Data was obtained on ten additional patients diagnosed to be vitamin B_{12} deficient and the total of 21 patients with vitamin B_{12} deficiency are shown in Table V. In each of the 21 patients the vitamin B_{12} values found when the RID assay was carried out utilizing human IF or hog IF were below the range of vitamin B_{12} values found in a control group of 74 normal subjects. However, when the RID assay was carried out utilizing human R or hog R only about half of the 21 vitamin B_{12} deficient patients were found to assay below the range of normal subjects for vitamin B_{12}. This indicates that where the newly found vitamin B_{12} analogues are present in the samples being tested, and the binding protein is not specific to vitamin B_{12}, the resulting assays may suggest that a truly vitamin B_{12} deficient patient is not within the deficient range. This may lead to delay of treatment of that patient for vitamin B_{12} deficiency. It also indicates that the vitamin B_{12} analogues that have now been discovered lack the therapeutic or beneficial activity of vitamin B_{12} in the sense of being unable to prevent the hematologic and/or neurologic abnormalities associated with vitamin B_{12} deficiency.

There are many commercial RID assay type kits available for the assay of vitamin B_{12} in clinical laboratories. Table VI sets forth an analysis of several such kits, and a comparison of the types of protein found in those kits with hog IF, hog R and hog IFC. By reference to Table VI, it is seen that the commercial kits appear to have only about 13% to about 35% intrinsic factor and from about 60% to about 85% R.protein. It is therefore suspected, that the use of these kits will give substantially erroneous assays of the amount of vitamin B_{12} present in a sample

Table VI

Analysis of vitamin B_{12} binders and assay pH used in commercial kits sold for the assay of vitamin B_{12} in clinical laboratories

Source of Binder	Vitamin B_{12} Binding Protein		Assay pH of Kit[d]
	intrinsic factor[a] (%)	R protein[b] (%)	
Hog IF	97	0	-
Hog R	0	98	-
Hog IFC	25	75	-
Diagnostic Products Corp. Kit	35	60	1.7
New England Nuclear Kit	20	82	4.1
Bio-Rad Laboratories Kit	30	71	1.9
Medvak Diagnostic Products Kit	13	85	1.8
Schwarz/Mann Kit	34	67	9.1
Pharmacia Diagnostics Kit	1 (33)[c]	67	4.1

(a) Inhibition of [^{57}Co] B_{12} binding observed with anti-IF antibodies at pH 7.5.
(b) Inhibition of [^{57}Co] B_{12} binding observed with a 100 fold molar excess of cobinamide at pH 7.5.
(c) In this kit the binder is covalently attached to an insoluble matrix and because of steric factors it may not be accesible to anti-intrinsic factor antibodies. Thus the value for % intrinsic factor may be as high as 33%.
(d) Refers to the pH measured after the addition of all of the components of each individual kit. R protein retains its full B_{12} binding ability over the range of pH 1.7-9.1 but intrinsic factor loses 10% and 99% of its B_{12} binding ability at pH 4.1 and 1.9, respectively. Thus kits that use pH 4.1 have slightly less % intrinsic factor than is indicated in the table while those that use pH 1.7-1.9 are employing essentially no intrinsic factor.

when the sample also includes vitamin B_{12} analogues, such as those newly discovered to exist in mammalian blood and tissues. It has also been determined that the effectiveness of intrinsic factor to bind vitamin B_{12} is somewhat pH dependent, with intrinsic factor losing about 10% of its binding ability at a pH of about 4.1 and losing about 99% of its binding ability at a pH of about 1.9. Thus, to the extent that the commercial kits use a pH of about 4.1 during binding they would have about 10% less intrinsic factor than shown in Table VI. Those kits having a pH during binding of about 1.7 to about 1.9 would obtain substantially no binding from intrinsic factor.

While the invention has been particularly shown and described with reference to preferred embodiments thereof, it will be understood by those skilled in the art that the foregoing and other modifications or changes in form and details may be made therein without departing from the spirit and scope of the invention.

What is claimed is:

CLAIMS

1. A composition for use in the assay of vitamin B_{12} (cobalamin) wherein the active component is substantially specific to vitamin B_{12} and substantially free of substances which react with vitamin B_{12} analogues.

2. The composition of claim 1 wherein the active component is a binding protein.

3. The composition of claim 2 wherein the binding protein is intrinsic factor.

4. The composition of claim 3 wherein the intrinsic factor is selected from the group consisting of human IF, hog IF and rabbit IF.

5. The composition of claim 4 wherein the intrinsic factor includes human IF.

6. The composition of claim 4 wherein the intrinsic factor includes hog IF.

7. The composition of claim 1 wherein one or more protein binder which is non-specific to vitamin B_{12} is present and has been rendered substantially inactive or inoperative as to its ability to bind with vitamin B_{12} by treatment with a vitamin B_{12} analogue, said analogue being substantially reactive with said non-specific protein and substantially non-reactive with the material which is a specific binder for B_{12}, said analogue being present in an amount sufficient to render substantially all of the non-specific binder inactive or inoperative in its ability to bind with vitamin B_{12}.

8. The composition of claim 7 wherein the specific active binder includes intrinsic factor protein and the non-specific protein binder includes an R protein.

9. The composition of claim 8 wherein a portion of the vitamin B_{12} analogue is selected from the group consisting of cobinamide, CN-Cbl(bde-OH) and (3,5,6-Me_3BZA) (CN,OH) Cba.

10. The composition of claim 7 wherein the vitamin B_{12} analogue is present in the amount of about a 10-fold to about a 100,000-fold excess, based on the molar amount of the non-specific protein.

11. The composition of claim 9 wherein the vitamin B_{12} analogue is present in the amount of about a 1-fold to about a 10,000,000-fold excess based on the molar amount of the non-specific protein.

12. The method of preparing the binder of claim 2 wherein a composition including binding material substantially specific to vitamin B_{12} and binding materials substantially non-specific to vitamin B_{12} and including a protein is treated with vitamin B_{12} analogues to remove substantially all of the non-specific vitamin B_{12} binding material, said vitamin B_{12} analogue being substantially reactive with said non-specific binder and substantially non-reactive with the material which is a specific binder for B_{12}, said analogue being added in an amount sufficient to render substantially all of the non-specific binder inactive or inoperative in its ability to bind with vitamin B_{12}.

13. The method of preparing the binder of claim 7 wherein a composition including protein binding material substantially specific to vitamin B_{12} and binding material substantially non-specific to vitamin B_{12} is treated with vitamin B_{12} analogue to render the binder non-specific to vitamin B_{12} substantially inactive or inoperative as to its ability to bind with vitamin B_{12}, said vitamin B_{12} analogue being substantially reactive with said non-specific binder and substantially non-reactive with the material which is a specific binder for B_{12}, said analogue being added in an amount sufficient to render substantially all of the non-specific binder inactive or inoperative in its ability to bind with vitamin B_{12}.

14. The method of claim 13 wherein the specific active binder includes intrinsic factor protein and the non-specific protein binder includes an R protein.

15. A radioisotope dilution assay for measuring the vitamin B_{12} level in a sample comprising:

contacting said sample with a known amount of a radioisotope of vitamin B_{12} and a composition containing a binding protein substantially specific to vitamin B_{12}, said composition being substantially free of substances which bind vitamin B_{12} analogues.

16. The method of claim 15 wherein the binding protein is intrinsic factor.

17. The method of claim 15 wherein the binding protein consists essentially of protein selected from the group consisting of human IF, hog IF and rabbit IF.

18. The method of claim 15 wherein the source of vitamin B_{12} is selected from the group consisting of mammalian tissue and mammalian blood.

19. The method of claim 15 wherein the source of vitamin B_{12} is human blood.

20. The method of measuring vitamin B_{12} analogues present in a sample which also includes vitamin B_{12}, including the steps of:

assaying a portion of the sample with a binder which is a binder for both vitamin B_{12} and vitamin B_{12} analogues;

assaying a portion of the sample with a binder which is substantially specific to vitamin B_{12} and substantially free of substances which bind vitamin B_{12} analogues; and

then determining the difference between the two assays as being substantially indicative of the amount of vitamin B_{12} analogue in the sample.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US79/00210

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. CL. G01N 33/16; U.S. CL. 23/230B, 230.3, 230.6; 252/408; 424/1, 1.5, 96

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
US	23/230B, 230.3, 230.6; 195/2; 252/408; 424/1, 1.5, 96

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X	US,A, 3,434,927 Published 25 March 1969, Highley et al.	1-11
X	US,A, 3,442,819 Published 06 May 1969, See Examples I-III, Herbert.	15-20
X	US,A, 3,937,799 Published 10 Feb. 1976, Lewin et al.	15-20
X	US,A, 3,591,678 Published 06 July 1971, Ellenbogen et al.	1-11
A	US,A, 2,892,754 Published 30 June 1959, Nimeguen.	1-11
A	US,A, 2,912,360 Published 10 Nov. 1959, Baum et al.	1-11
A	US,A, 3,980,764 Published 14 Sept. 1976, Adams.	1-11
A,P	US,A, 4,133,951 Published 09 Jan. 1979, Charlton et al.	1-11
A,P	US,A, 4,136,159 Published 23 Jan. 1979, Stone.	1-11
A,P	US,A, 4,146,602 Published 27 March 1979, Gutcho et al.	1-11
A	GB,A, 771,068 Published 27 March 1957.	1-11
A	GB,A, 791,186 Published 26 Feb. 1958.	1-11
A	GB,A, 900,459 Published 04 July 1962.	1-11

* Special categories of cited documents: [15]

"A" document defining the general state of the art

"E" earlier document but published on or after the international filing date

"L" document cited for special reason other than those referred to in the other categories

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but on or after the priority date claimed

"T" later document published on or after the international filing date or priority date and not in conflict with the application, but cited to understand the principle or theory underlying the invention

"X" document of particular relevance

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
10 July 1979	17 JUL 1979

International Searching Authority [1]	Signature of Authorized Officer [20]
ISA/US	Sidney Marantz SIDNEY MARANTZ

Form PCT/ISA/210 (second sheet) (October 1977)